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Acetylcholinesterase and Acetylcholine Receptor

Annual Report

Saul G. Cohen, Ph.D.

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We seek information about the properties and amino acid composition of the active site of acetylcholinesterase (AcChE). Work is based on the view that the part at which the positively charged β-trimethylammonio substituent of acetylcholine (AcCh) binds is not anionic but is uncharged and complementary to the trimethyl character of that substituent. The subsite would be more specifically explored by uncharged reagents, while positive reagents would react with peripheral anionic groups. AcChE was isolated from Torpedo nobiliana by affinity chromatography, gel electrophoresis, and autoradiography after ^{3}H-DFP labeling showed satisfactory purity and the major band at ~70 kD. Sigma eel AcChE showed much extraneous protein, and was

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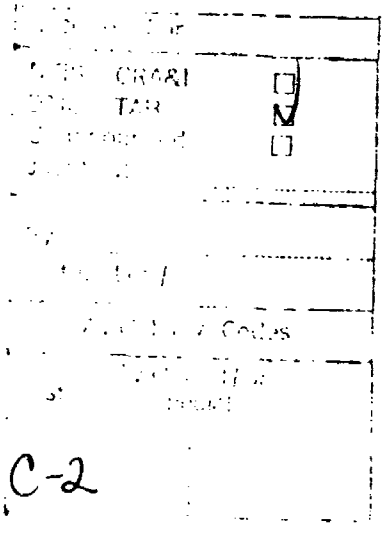
19. ABSTRACT(cont.)

suitable for kinetic studies but not for study of amino acid content. Initial emphasis is on labeling with 1-^{14}C-1-bromopinacolone, $(CH_3)_3CCO^{14}CH_2Br$, which will be prepared in 60 μmole quantity. Other potential irreversible labeling agents were studied briefly: chloromethyl pivalate, $(CH_3)_3CCO_2CH_2Cl$, chloromethyl acetate, $CH_3CO_2CH_2Cl$, methyl trimethylammonioacetate, $(CH_3)_3N^+CH_2CO_2CH_3$, tert-butyl peracetate, $(CH_3)_3C$-$OOCOCH_3$, methyl methanesulfonate, $CH_3SO_2OCH_3$, methyl benzenesulfonate, $C_6H_5SO_2OCH_3$, and styrene epoxide, $C_6H_5CH(O)CH_2$. The methylsulfonyl and trichloromethyl groups were examined in reversible inhibitors, methylsulfonylethyl acetate, $CH_3S(O_2)$-$CH_2CH_2OCOCH_3$, trichloroethanol, Cl_3CCH_2OH, and chloral hydrate, $Cl_3CCHO\cdot H_2O$. Nitrobenzene was an effective reversible inhibitor and polar interactions at the aryl binding site will be studied.

A study of hydrolysis of β-substituted ethyl acetates, β-X-$CH_2CH_2OCOCH_3$, by AcChE was published. Reactivity, normalized for effect of the β-X-substituent on intrinsic hydrolytic activity, rose linearly with volume of the X-substituent, X = H, Cl, Br, CH_3CH_2, $(CH_3)_2CH$, $(CH_3)_2S^+$, $(CH_3)_3N^+$, $(CH_3)_3C$; the trimethyl subsite accommodates the larger $(CH_3)_3Si$ substituent with no further increase in activity. β-X-Substituents with polar surfaces, $CH_3S(O_2)$ and $(CH_3)_2N^+O^-$, showed lower reactivity than consistent with volume.



Summary

This research seeks information about the properties and amino acid content of the active site of acetylcholinesterase (AcChE). It is based largely on the view that the subsite at which the positively charged β-substituent of the natural substrate, acetylcholine (AcCh), binds is not anionic, as is generally accepted, but uncharged and complementary to the trimethyl substituted character of that substituent. The subsite would be more specifically explored by uncharged reagents, while positive reagents would react at peripheral negative sites. Thus, uncharged irreversible and reversible inhibitors and substrates are obtained or synthesized and their reactions with the enzyme are studied. Primary emphasis is placed on labeling the active site with 1-^{14}C-1-bromopinacolone, $(CH_3)_3C\text{-}CO^{14}CH_2Br$ (^{14}C-BrPin); we have found in previous work that BrPin inactivates AcChE with the same kinetics toward hydrolysis of substrates of widely varied structure.

Keywords. Torpedo nobiliana; chromatography; electrophoresis

In the current contract year equipment was assembled and AcChE was isolated from Torpedo nobiliana by affinity chromatography; gel electrophoresis showed a major band at ~70 kD, other bands at 124 and 103 kD, quite faint bands at 33, 50 and 45 kD. A sample was labeled with ^{3}H-diisopropylfluorophosphonate (DFP) and showed radioactivity only at 66-67 kD. Synthesis of ^{14}C-BrPin in micromole quantity will be worked on. Purchased Sigma eel AcChE was found to contain much extraneous protein and degraded enzyme subunit, ~50 kD. It is satisfactory for kinetic studies, with acetylcholine controls, but isolated Torpedo enzyme will be used for characterization.

Other potential irreversible inhibitors were surveyed briefly in enzymic hydrolysis of AcCh. Chloromethyl pivalate, $(CH_3)_3CCO_2CH_2Cl$, closely related to BrPin, inactivated to an extent similar to BrPin. Chloromethyl acetate, $CH_3CO_2CH_2Cl$, which probably binds at the esteratic or acetyl subsite, appeared to act both as a weak substrate, ~1% as reactive as AcCh, and as an irreversible inhibitor, similar in activity to BrPin. Trimethylammoniomethyl acetate, $(CH_3)_3N^+CH_2OCOCH_3$, the lower homologue of AcCh, acted as a reversible inhibitor, K_I = 1 mM, as a substrate, ~10% as reactive as AcCh, and as a weak irreversible inhibitor, possibly by alkylation or by formation of formaldehyde in the active site. tert-Butyl peracetate, $(CH_3)_3COOCOCH_3$, acted as both a substrate, ~10% as reactive as AcCh, and as an irreversible inhibitor, possibly by generating tert-butyl hydroperoxide, $(CH_3)_3COOH$, in the active site.

The methanesulfonyl group, $CH_3S(O_2)$, similar in shape to tert-butyl, $(CH_3)_3C$, appears to lead to similar binding when examined in methylsulfonylethyl acetate, $CH_3S(O_2)CH_2CH_2OCOCH_3$, both as a substrate and as a reversible inhibitor. In methyl methanesulfonate, $CH_3SO_2OCH_3$, it leads to rapid irreversible inhibition, probably by alkylation; methylsulfonyl chloride,

CH_3SO_2Cl, inactivates like methylsulfonyl fluoride, probably by acylating the serine hydroxyl. The CH_3SO_2 group in reversible inhibitors may lead to useful water and oil permeable moderators of AcChE activity.

The trichloromethyl group in trichloroethanol, Cl_3CCH_2OH, leads to weak binding similar to that of its carbon analogue, neopentyl alcohol, $(CH_3)_3CCH_2OH$, K_I = ~40 and 30 mM, respectively. Chloral hydrate, $Cl_3CCH{=}O{\cdot}H_2O$, K_I = 1 mM, binds more strongly than its carbon analogue, pivalaldehyde, $(CH_3)_3CCH{=}O$.

Examination of an aryl binding subsite was begun. Methyl benzenesulfonate, $C_6H_5SO_2OCH_3$, K_I = 6 mM, rapidly inactivates irreversibly, and this inactivation is not retarded by tetramethylammonium ion. Styrene epoxide, K_I = 10 mM, inactivates AcChE less rapidly. Nitrobenzene is an effective reversible inhibitor, K_I = 0.5 mM, in hydrolysis of AcCh, while, like small cations, it accelerates hydrolysis of ethyl acetate. Further studies will be carried out on the charge-transfer character of the aryl binding subsite, and on development of labeling agents for it.

A study of hydrolysis of β-substituted-ethyl acetates, β-$X\text{-}CH_2CH_2OCOCH_3$, by AcChE was completed and published. Reactivity, normalized for effect of the β-substituent on intrinsic hydrolytic reactivity toward hydroxide, log $(k_{cat}/K_m)_n$, rises linearly with volume of the substituent, as measured by molar refractivity (MR) for β-X = H, Cl, Br, CH_3CH_2, $(CH_3)_2CH$, $(CH_3)_2S^+$, $(CH_3)_3N^+$ and $(CH_3)_3C$; reactivity levels off with the latter, while the trimethyl site accommodates the larger $(CH_3)_3Si$ group with no further increase in reactivity. β-Substituents with polar surfaces, $CH_3S(O_2)$ and $(CH_3)_2N^+O^-$, show lower reactivity than is consistent with their MR values, possibly because they do not remove H_2O from the active site. The correlation with MR is superior to that with hydrophobicity, π, which predicts low reactivity for the important positively charged substrates. Study of substrates will probably not be extended.

Collaboration continues with Professor J. B. Cohen, Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis. Compounds for his study of the AcCh receptor are prepared at Brandeis; labeling of AcChE is carried out at Brandeis; and final characterization of labeled peptides will be carried out at Washington University.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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(1) Statement of the Problem Under Study

We seek information about the properties of the active site of acetylcholinesterase (AcChE) by study of the response of the enzyme to substrates and inhibitors of varied structure. The work is based on our view that the subsite at which the positively charged group of acetylcholine (AcCh) binds is not anionic, but is uncharged and better considered trimethyl, complementary to the trimethyl property of that substituent. We consider that this subsite may be irreversibly labeled more specifically by reaction with a trimethyl-substituted alkylating agent, 1-bromopinacolone (BrPin), $(CH_3)_3CCOCH_2Br$.

We have isolated AcChE from Torpedo nobiliana, purified it by affinity chromatography, labeled it with ^{3}H-diisopropylfluorophosphonate (DFP), subjected it to gel electrophoresis, staining and autoradiography. Distinct bands were found on staining, and radioactivity was found at 66-67 kD. Purchased Sigma eel AcChE showed many protein bands on gel electrophoresis and ^{3}H-DFP label at 49 and 52 kD. Purified Torpedo enzyme will be used in characterization of the active site; Sigma enzyme may be used for kinetic studies.

Other active site-directed neutral and cationic irreversible inhibitors are being developed: chloromethyl pivalate, $(CH_3)_3CCO_2CH_2Cl$; chloromethyl acetate, $CH_3CO_2CH_2Cl$; trimethylammoniomethyl acetate, $(CH_3)_3N^+CH_2OCOCH_3$; and tert-butyl peracetate, $(CH_3)_3COOCOCH_3$, potential alkylating agents and/or suicide inhibitors. The trimethyl site was further explored with inhibitors and substrates containing substituents similar to trimethyl groups in shape and volume but differing in surface properties, with Cl and O replacing methyl: methyl methanesulfonate (MMS), $CH_3SO_2OCH_3$, methylsulfonyl chloride (MSC), CH_3SO_2Cl, methylsulfonylethyl acetate, $CH_3SO_2CH_2CH_2O$-$COCH_3$, trichloroethanol, Cl_3CCH_2OH, and chloral hydrate, $Cl_3CCH{=}O{\cdot}H_2O$. These compounds, except the last three, may be useful labeling agents. BrPin remains preferred at present for specific study of the trimethyl subsite, and will be prepared with ^{14}C-label. Benzene derivatives were studied briefly as reversible and irreversible inhibitors of AcChE; effects of electron donating and withdrawing substituents will be studied for information as to the nature of the binding interaction at an aryl binding subsite.

(2) Background and Review of Appropriate Literature

Although the part of the active site of AcChE binding the trimethylammonium group of AcCh, $(CH_3)_3N^+CH_2CH_2OCOCH_3$, has generally been considered as anionic (1-3), our recent studies

indicate that it may not contain a specific negative charge, and may be better considered trimethyl, as complementary to the trimethyl-substituted character of the β-substituent rather than to its positive charge (4). This was proposed when the enzymic reactivity of a series of β-substituted ethyl acetates, $X\text{-}CH_2CH_2OCOCH_3$, with cationic, uncharged polar and non-polar substituents was satisfactorily accounted for by two properties of the β-substituents: (i) their effects on the intrinsic reactivity as measured by hydrolysis by hydroxide, and (ii) their apparent molar volumes, $\bar{V}^{\circ}_{25}$. This is indicated in eq. 1,

$$\log(k_{2(n)}/K_s) = a\bar{V}^{\circ}_{25} + C \qquad (1)$$

where $\log (k_{2(n)}/K_s)$ is the enzymic reactivity normalized for intrinsic hydrolytic reactivity. Normalized reactivity, indicated by $k_{2(n)}$ or $(k_{cat}/K_m)_n$, is the experimentally observed value multiplied by the ratio of rate constants for hydrolysis of AcCh by OH^- to that for the acetate substrate being studied. The effect of cationic charge in X is to increase intrinsic reactivity by a factor of about 25 as compared with that for analogous alkyl substituents, and substantial effects of the postulated (5) specific anionic site in attracting and orienting cationic substrates are not required (4).

The enzyme has isoelectric point ~pH 5 (6,7), and thus has excess negative charge on its surface at pH 7-8. Cationic reversible inhibitors structurally related to AcCh bind better than their uncharged analogues by small factors, corresponding to ~1 kcal/mole of binding energy, much less than would be caused by the interaction of $(CH_3)_3N^+$ with a "contact" anionic O^- that is implied by a specific anionic site (8). Moreover, ionic strength effects on binding and on hydrolysis have been interpreted in terms of anionic charges on the enzyme surface peripheral to the active site (9). Each of the inhibitors studied was structurally related to AcCh and, whether neutral or cationic, showed essentially the same binding constant when retarding hydrolysis of AcCh and its uncharged analogue, 3,3-dimethylbutyl acetate. This indicated that the β-trimethylammonio and β-tert-butyl groups of the two substrates and of the related inhibitors bind at the same subsite (8). The substrate study had indicated that this subsite is complementary to uncharged trimethyl, and the properties of the subsite would then be more specifically explored with uncharged reagents (4). Indeed, arylaziridinium reagents, intended to alkylate the "anionic" site and prevent substrate access, inhibited completely the hydrolysis of cationic but not of neutral esters (10,11). We took this to indicate not that there are separate anionic and neutral subsites, but that cationic agents act at peripheral anionic groups, thus increasing positive charge and repelling cationic substrates and inhibitors; they modify the active site domain but allow

uncharged substrates to bind at the one trimethyl site and react. This view was borne out in studies with 1-bromopinacolone, $(CH_3)_3CCOCH_2Br$ (BrPin), which inhibited AcChE irreversibly with the same effect on hydrolysis rates of a variety of both cationic and neutral substrates. BrPin apparently exerted its inhibitory effect via binding at the trimethyl site (12).

(3) Rationale For the Current Study

The results indicating that AcChE inactivation by BrPin appears to involve BrPin interaction with the active site suggest these studies to explore the AcChE active site:

(i) isolation and labeling of AcChE from Torpedo nobiliana; receptor studies are carried out with electric organ from Torpedo nobiliana at Brandeis and at Washington University, and frozen organ is readily available locally.

(ii) a program to characterize the active site by labeling amino acid(s) in it with radioactive BrPin,

(iii) attempts to prepare other irreversible inhibitors, potential labeling reagents based on the tert-butyl group,

(iv) exploration of the active site with acetate substrates containing varied β-substituents -- $(CH_3)_3Si$-, $(Cl_3)C$-, $(CH_3)_2S^+$-, $CH_3S(O_2)$-, $(CH_3)S(O)$-, $(CH_3)_2N(O^-)$- -- and with structurally related reversible and irreversible inhibitors,

(v) study of uncharged benzene derivatives as reversible and irreversible inhibitors, and as accelerators.

(4) Experimental Methods

Isolation of Acetylcholinesterase.

Frozen electric organ of Torpedo nobiliana was obtained from Sal Testaverde, Gloucester, Mass., and stored at -70°C. Literature procedures for AcChE isolation were adapted (7,13) with purification by affinity chromatography (14,15).

The affinity ligand was prepared in accordance with literature procedures (14,15) by the reaction series: acylation of 6-aminohexanoic acid with benzyl chloroformate, condensation of this product with m-aminodimethylaniline in the presence of dicyclohexylcarbodiimide (DCC), quaternization with methyl iodide, and cleavage of the carbobenzyloxy group with HBr, eqs. 2-5.

$$H_2N(CH_2)_5CO_2H + C_6H_5CH_2OCOCl \rightarrow C_6H_5CH_2OCONH(CH_2)_5CO_2H \quad (2)$$

$$C_6H_5CH_2OCONH(CH_2)_5CO_2H + m\text{-}(CH_3)_2NC_6H_4NH_2$$

$$\downarrow \text{DCC} \quad (3)$$

$$m\text{-}(CH_3)_2NC_6H_4NHCO(CH_2)_5NHCO_2CH_2C_6H_5$$

$$\downarrow + CH_3I \quad (4)$$

$$m\text{-}(CH_3)_3N^+C_6H_4NHCO(CH_2)_5NHCO_2CH_2C_6H_5$$

$$\downarrow + HBr \quad (5)$$

$$m\text{-}(CH_3)_3N^+C_6H_4NHCO(CH_2)_5NH_2 \cdot HBr$$

The ligand was coupled to 6-aminohexanoic acid-Sepharose B by 1-(3-dimethylaminopropyl-3-ethyl)carbodiimide. The affinity column was tested with Sigma electric eel AcChE, which was bound satisfactorily and eluted with 1 mM decamethonium; 120 units were applied to the column and 99 units were recovered. Enzyme was assayed by the Ellman procedure (16).

Sodium dodecyl sulfate (SDS) polyacrylamide gel equipment was assembled and procedures for assay of AcChE labeled with ^{3}H-DFP were carried out (17,18). Slab gels were 8% polyacrylamide, 1.5 mm thick; stacking gels were 4% polyacrylamide. Electrode buffer contained 0.04 M Tris, 0.38 M glycine, 0.1 mM sodium thioglycolate and 1.5 g/L SDS. This composition led to pH 8.3 and no additional buffer was used. Staining solution was 1.25 g Coomassie blue in 500 mL of 1:1:0.2 methanol, distilled water, acetic acid. Destaining solution was 25% methanol, 10% acetic acid in distilled water. Staining and destaining were carried out at room temperature. Sigma SDS markers were used: myosin, M.W. 205,000; β-galactosidase, 116,000; phosphorylase b, 97,000; bovine albumin, 66,000; egg albumin, 45,000; and carbonic anhydrase, 29,000.

Labeling and electrophoresis were examined with Sigma AcChE. An initial large amount was taken, since the enzyme content appeared low: 620 μg in 150 μL of buffer I (0.1 M NaCl, 40 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 8.0) was divided into 2 portions; to 1 was added 2 μL of 50 mM decamethonium, and both were treated with 30 μL of 5.7μM (0.9 μCi) of ^{3}H-DFP at 4°C for 1 hr. β-Mercaptoethanol, 20 μL, was added to 1 mL of a stock solution of 40% sucrose, 10% SDS, 0.3 M Tris (pH 6.8), 0.0125% bromophenol blue, and 2% glycerol; 25 μL of this was added to 105 μL of the enzyme solution, and this was heated at 100°C for

2 min. Portions of the dissociated enzyme were loaded onto the stacking gel and electrophoresis was carried out at 100 V for 5-6 hr, with the markers on a separate track. The gel was prefixed for 20 min in a solution of 50% methanol, 10% acetic acid, and 40% distilled water, stained with Coomassie blue for 30 min, destained overnight, treated with Enhance for 1 hr and with distilled water for 1 hr and dried in a Biorad gel dryer for 2 hr at 68°C. Stain bands were found at 81, 73, 52, 49, 37, and 29 kD. Streaking of protein on the gel was observed. The Sigma AcChE, used as received for examination of the labeling and electrophoresis, appeared to contain a range of protein fractions. The gel was placed in contact with preexposed Kodak X-Omat film for 43 hr at -70°C and developed. The solution treated with ^{3}H-DFP in the presence of decamethonium showed no label; the one without decamethonium showed ^{3}H-DFP label at 52 and 49 kD. The active fraction, reacting with DFP, appeared somewhat degraded, as compared with an expected subunit of ~70 kD. The equipment and procedure appeared satisfactory and attention was turned to isolation and purification of AcChE.

Electric organ tissue, ~500 g, was thawed at 4°C, suspended in ~1500 mL of buffer I, homogenized for four 1 minute periods at 4°C and centrifuged for 2 hr at 7000 rpm in a 6 x 250 mL rotor. The pellet was resuspended in 250 mL of buffer I, treated with 5 μg/mL of Sigma trypsin for 10 min at 37°C, and then with 10 μg/mL of Sigma Type I-S soybean trypsin inhibitor. The suspension was cooled to 4°C, centrifuged for 90 min at 25 K in a 12 x 25 mL rotor, applied to a 12 x 1.5 cm affinity column, and eluted at 37 mL/hr, 4°C. The column was washed with buffer I until clear of protein and assayed at 280 nm. Enzyme was eluted with 1 mM decamethonium in buffer I; protein and activity were measured (16). Active fractions were pooled and decamethonium was removed on a 15 x 1.5 cm C-2-120 CM Sephadex column; the eluate was concentrated to 5 mL by ultrafiltration, dialyzed against three 1 L portions of buffer I for 48 hr, further concentrated to 450 μL (Amicon micro-ultrafilter) and stored at -70°C in 100 μL aliquots. The concentrated solution was assayed for protein with a Biorad assay kit and for enzyme content by hydrolysis of acetylthiocholine (16). All steps were carried out at 4°C unless otherwise noted.

Kinetic Procedures.

Kinetic studies were carried out with pH stat equipment as described in our previous publications (4,8). Exploratory study of potential irreversible inhibitors was carried out as in the study of inhibition by BrPin (12). Inhibitors containing acetate ester functions were also examined briefly as substrates (4). Reversible inhibitors were studied as described previously (8).

(5) Results

Isolation and Labeling of AcChE.

Affinity ligand and an affinity column were prepared and tested. Gel electrophoresis equipment was assembled and tested with ^{3}H-DFP-labeled Sigma eel AcChE. Frozen electric organ of Torpedo nobiliana was obtained; three 500 g portions were used for enzyme isolation. AcChE was obtained in concentrated solution by affinity chromatography and decamethonium elution.

Table I

Isolation of Torpedo Acetylcholinesterase

Preparation	Volume μL	AcChE Units μmole AcSCh/min[a]	Protein mg	AcChE unit/mg
(1)	230	7860	6.44	1220[b]
(2)	320	9080	5.56	1650[b]
(3)	450	7800	4.18	1866

[a] AcSCh, acetylthiocholine
[b] These samples contain decamethonium.

An aliquot of solution 3 above, 10 μL (93 μg protein) diluted to 30 μL with phosphate buffer, pH 7, incubated with 8 μL of 5.7 μM ^{3}H-DFP and subjected to electrophoresis and autoradiography, showed a broad heavily stained band at 66-72 kD, other bands at 103 and 124 kD, and faint bands at 45, 50, and 133 kD. Little stain was seen at the dye front, indicating little degradation. Use of less protein will give better resolution. ^{3}H-DFP label was observed only at the major, broad band, at 66-67 kD. The value of k_{cat} for AcChE from Torpedo californica (3.3×10^5 min^{-1}) (19), if valid for Torpedo nobiliana, leads for solution 3, above, to $(7.8 \times 10^{-3})/(3.3 \times 10^5) = 2.4 \times 10^{-8}$ mole of AcChE, equal to 1.6 mg (m.w. = 67000). Thus, solution 3 contains 1.6 mg of AcChE in 4.18 mg of protein, or ~40% enzyme.

In one solution, salt regeneration of the column after elution with decamethonium led to recovery of additional enzyme, 1270 units in 4.61 mg, 275 units/mg. This material subjected to labeling and electrophoresis showed a minor band

at 99 and a major band at 73 kD, and only the latter was labeled by ^{3}H-DFP.

Preliminary experiments were carried out on AcChE as it was being isolated from Torpedo. Trypsin-released enzyme, before purification by affinity chromatography, was completely inactivated by 3 mM BrPin in 4 hr, while Sigma eel AcChE was ~50% inactivated. On the other hand, as a competitive reversible inhibitor, BrPin had K_I = 0.7 mM for Torpedo AcChE, 0.2 mM for eel enzyme. 4-Oxo-N,N,N-trimethylpentaminium, $(CH_3)_3N^+CH_2-CH_2CH_2COCH_3$, had K_I = 0.2 mM for Torpedo, 0.08 mM for eel enzyme. Hydrolysis of AcCh led to K_m = 0.05 mM for Torpedo, 0.3 mM for eel enzyme. Column-purified enzyme will be characterized.

Other Potential Irreversible Inhibitors.

Chloromethyl pivalate, $(CH_3)_3CCOOCH_2Cl$ (Aldrich), 3.5 mM, structurally analogous to BrPin, led to complete inactivation after 3 hr when the incubating solution was allowed to become acidic; 50% inactivation was observed when pH was maintained at 7.8. This compound, examined as a substrate, appeared to hydrolyze slowly.

Chloromethyl acetate, $CH_3CO_2CH_2Cl$, was synthesized from formaldehyde and acetyl chloride (20). At 4.4 mM it led to 90% inactivation after 4 hr when the incubation solution was allowed to become acidic, and to 70% inactivation when pH was maintained at pH 7.8. As a substrate it showed k_{cat}/K_m ~10^4 $sec^{-1}/16$ mM = 6 x 10^5 M^{-1} sec^{-1}, 4 x 10^5 M^{-1} sec^{-1} when normalized for its high intrinsic hydrolytic reactivity; $k_{(OH^-)}$ = 4.4 M^{-1} sec^{-1}.

No reactivation occurred in either case on standing or on treatment with 2-methyl-pyridinium aldoxime (PAM).

Trimethylammoniomethyl acetate, $(CH_3)_3N^+CH_2OCOCH_3$ (21), examined as a reversible inhibitor, showed mixed competitive (comp) and noncompetitive (nonc) inhibition, $K_{I(comp)}$ = 1.4 mM, $K_{I(nonc)}$ = 4.8 mM. As a substrate it had k_{cat}/K_m = 3.9 x 10^3 $sec^{-1}/0.8$ mM = 4.5 x 10^6 M^{-1} sec^{-1}, 3.8 x 10^6 M^{-1} sec^{-1} when normalized for intrinsic reactivity; $k_{(OH^-)}$ = 3.3 M^{-1} sec^{-1}. After hydrolysis of 3 mM of this compound, reactivity of the enzyme was reduced ~30%.

tert-Butyl peracetate, $(CH_3)_3COOCOCH_3$ (Pfaltz and Bauer), is hydrolyzed by AcChE, k_{cat}/K_m = 3.4 x 10^3 $sec^{-1}/2.8$ mM = 1.2 x 10^6 M^{-1} sec^{-1}, 1.7 x 10^6 M^{-1} sec^{-1} when normalized for its intrinsic hydrolytic reactivity; $k_{(OH^-)}$ = 2.0 M^{-1} sec^{-1}. The measured enzymic activity was greater than that of its carbon non-peroxidic analogue neopentyl acetate, $(CH_3)_3CCH_2OCOCH_3$, k_{cat}/K_m = 1.9 x 10^2 $sec^{-1}/8.6$ mM = 2.2 x 10^4 M^{-1} sec^{-1}, but normalized activities were similar, 8.8 x 10^5 M^{-1} sec^{-1} for the

neopentyl compound. Enzymic hydrolysis (4 nM enzyme) of 3 mM peracetate was complete in 1 hr, when enzymic reactivity was decreased ~30%. Further additions of two portions of 3 mM peracetate led to 50 and 30% residual activity. Similar treatments with 2 mM tert-butyl hydroperoxide (Aldrich) led to smaller decreases in reactivity, 10, 15 and 25%.

Methyl methanesulfonate (MMS), $CH_3SO_2OCH_3$, (Aldrich). This alkylating agent, at 9 mM, inactivated rapidly, 40% in 1.6 hr, completely in 4 hr, in hydrolysis of both AcCh and 3,3-dimethylbutyl acetate. At 1 mM no inhibition was observed for 4 hr, then 50% inactivation was found after 6 hr. At 3 mM there was 30% inactivation in 1 hr, 70% in 2 hr. Tetramethylammonium ion, 30 mM, and 4-oxo-N,N,N-trimethylpentaminium, 0.2 mM, afforded complete protection for 1 hr and then inactivation proceeded. As a reversible inhibitor, MMS acted purely non-competitively, K_I = 96 mM.

Methyl benzenesulfonate (MBS), $C_6H_5SO_2OCH_3$ (Pfaltz and Bauer), showed mixed reversible inhibition, $K_{I(comp)}$ = 6 mM, $K_{I(nonc)}$ = 21 mM, and irreversible inactivation. At 3 mM, there was 65% inactivation in 1 hr, 95% in 2 hr, largely unaffected by 30 mM tetramethylammonium ion. At 0.6 mM there was no inactivation after 1 hr, 15% after 3.3 hr, 33% after 5 hr, 93% after 24 hr, with similar effects in hydrolysis of both AcCh and 3,3-dimethylbutyl acetate.

Methylsulfonyl chloride (MSC), CH_3SO_2Cl (Aldrich), at 3 mM, inactivated AcChE completely in 10 min; at 0.3 mM, it inactivated 54% in 10 min, and inactivation was complete after 19 hr. At 0.03 mM there was 38% inactivation after 10 min, 72% after 14 hr. MSC appears as active as methylsulfonyl fluoride (MSF), CH_3SO_2F.

Reversible Inhibitors.

Methylsulfonylethyl acetate, $CH_3SO_2CH_2CH_2OCOCH_3$ (22), previously studied as a substrate, k_{cat}/K_m = 1.1 x 10^3 sec^{-1}/0.2 mM (23), was studied as a reversible inhibitor for hydrolysis of AcCh and found to have $K_{I(comp)}$ = 6.4 mM, $K_{I(nonc)}$ = 11 mM.

5-Dimethylamino-2-pentanone-N-oxide, $(CH_3)_2N^+(O^-)CH_2CH_2$-CH_2COCH_3 (synthesized), shows competitive reversible inhibition, K_I = 14 mM, very similar to binding of the corresponding ester substrate, $(CH_3)_2N^+(O^-)CH_2CH_2CH_2OCOCH_3$, K_m = 18 mM (23).

Trichloroethanol, Cl_3CCH_2OH (Aldrich), $K_{I(comp)}$ = 42 mM, $K_{I(nonc)}$ = 110 mM, inhibits similarly to its trimethyl analogue, neopentyl alcohol, $(CH_3)_3CCH_2OH$ (Aldrich), $K_{I(comp)}$ = 29 mM, $K_{I(nonc)}$ = 74 mM, and to the ether isomer of the latter,

methyl t-butyl ether, $(CH_3)_3COCH_3$ (Aldrich), $K_{I(comp)}$ = 25 mM, $K_{I(nonc)}$ = 24 mM.

Chloral hydrate, $Cl_3CCH{=}O \cdot H_2O$ (Aldrich), is an effective competitive reversible inhibitor, K_I = 1 mM, binding substantially more strongly than its trimethyl analogue, pivaldehyde, $(CH_3)_3CCH{=}O$ (Aldrich), K_I = 10 mM.

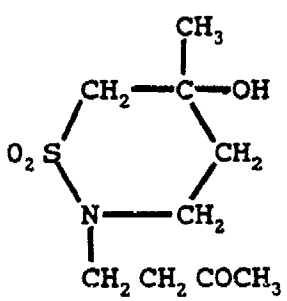
In collaboration with Professor J. B. Cohen, Washington University School of Medicine, St. Louis, the enantiomers of diethylaminoethyl α-phenylbutyrate, (+) and (-) $CH_3CH_2CH(C_6H_5)$-$COOCH_2CH_2N(C_2H_5)_2$, were prepared.

Addition of N,N-dimethyl- and N,N-diethylhydroxylamines to methylvinyl ketone has not been successful:

$$R_2NOH + CH_2{=}CHCOCH_3 \not\longrightarrow R_2NOCH_2CH_2COCH_3 \qquad (6)$$

Addition of methanesulfonamide, which shows no reversible inhibition, to methylvinyl ketone, was intended to form a 1:1 adduct but appeared to form a 1:2 adduct which then cyclized:

$$CH_3SO_2NH_2 + 2CH_2{=}CHCOCH_3 \rightarrow CH_3SO_2N(CH_2CH_2COCH_3)_2 \rightarrow$$



(7)

Benzene Derivatives as Inhibitors and Accelerators.

Styrene epoxide (Aldrich) showed mixed reversible inhibition, $K_{I(comp)}$ = 10 mM, $K_{I(nonc)}$ = 33 mM. At 3 mM it caused progressive irreversible inactivation, ~50% in 6 hr, 90% in 23 hr.

Attempts to convert phenacyl bromide, $C_6H_5COCH_2Br$, to its enol acetate by reaction with isopropenyl acetate failed.

Nitrobenzene, previously found to be an efficient reversible mixed inhibitor in the hydrolysis of AcCh, K_I = 0.5 mM, appears to be slightly less effective in inhibiting hydrolysis of 3,3-dimethylbutyl acetate, K_I = 0.8 mM, and still less effective towards n-butyl acetate, 2.1 mM, and 2-methylsulfonylethyl acetate, 2.8 mM.

Nitrobenzene accelerates enzymic hydrolysis of ~0.1 M ethyl acetate by decreasing K_m; 1 mM nitrobenzene doubles the rate, and 2 and 3 mM increase it fourfold. Acetophenone, 7 mM, accelerates hydrolysis of ethyl acetate threefold, while benzonitrile is less effective, 7 mM leading to 50% acceleration of ethyl acetate hydrolysis.

Substrates.

In an extension of the study of the range of applicability of eq. 1, the relation of enzymic reactivity to volume of β substituent, an alternative measure of volume, refraction volume, MR (24-27), which is available for a broader range of structures, was substituted for apparent molal volume $\bar{V}^{\circ}_{2,5}$. Results, largely obtained prior to the current contract, are summarized in Table II and Figure 1. The increase in normalized enzymic reactivity with volume leveled off with AcCh and 3,3-dimethylbutyl acetate. The larger analogous silyl compound, $(CH_3)_3SiCH_2CH_2OCOCH_3$, was accommodated in the active site with no substantial increase in reactivity. The methylsulfonyl, CH_3SO_2; dimethylamine oxide, $(CH_3)_2N^+(O^-)$; methylsulfoxy, $CH_3S(O)$; and methylthio, CH_3S substituents led to substantially lower reactivity than is consistent with MR values. In correlation with hydrophobicity (π), a structural parameter widely used in drug design (24,28-31), these substituents (except for methylthio) and the important cationic substituents $(CH_3)_3N^+$ and $(CH_3)_2S^+$, showed substantially greater reactivity than is consistent with π values (Figure 2). It is noteworthy that the hydrophilic CH_3SO_2 substrate, Compound IV (Table II), binds similarly to the hydrophobic $(CH_3)_3C$ substrate, Compound II A, K_m = ~6 mM, with substantially lower k_{cat} and greatly lower normalized reactivity (23).

(6) Discussion and Conclusions

A source of electric organ of Torpedo nobiliana is at hand, and procedures have been worked out for isolation of AcChE from this tissue by affinity chromatography in yields of ~10 mg/kg. This is less than but comparable to reported (7,13) yields from Torpedo californica. Purity of the enzyme appears satisfactory for labeling with BrPin. Sigma eel AcChE contains much low molecular weight material. Preliminary experiments on crude Torpedo enzyme, not purified by chromatography, indicate rapid inactivation by BrPin. Properties of purified enzyme, i.e., K_m for AcCh and K_I for standard inhibitors, will be evaluated. BrPin remains the preferred uncharged reagent for active site labeling. Conditions must be established for preparation of ^{14}C-labeled BrPin in μmole quantity. Additional purification of the enzyme before labeling will also be considered.

Other irreversible inhibitors are being prepared and examined. Chloromethyl pivalate, $(CH_3)_3CCOOCH_2Cl$, is a potentially useful reagent. At 3.5 mM, at pH 7.8, it inactivates with the same effectiveness as 3 mM BrPin, 50% in 3 hr. It is not clear at present whether greater apparent effectiveness when the solution is allowed to become acidic is due to less destruction of the inhibitor or lower stability of the enzyme at acid pH. This may be checked with proper control. Enzyme inactivated at pH 7.8 is not regenerated by PAM, indicating that the active site serine may not be acylated and that alkylation by the chloromethyl group occurs, as with the structurally analogous BrPin. Long standing at pH 7.8 does not regenerate activity, indicating that the alkylation may not be followed readily by hydrolysis of the ester group. This compound may bind at the trimethyl site as BrPin does, and in alkylating, react with the same or a different nucleophile; it merits further study.

Chloromethyl acetate, $CH_3CO_2CH_2Cl$, inactivates the enzyme at pH 7.8 with efficiency similar to that of BrPin and chloromethyl pivalate, and also shows greater effectiveness when the solution is allowed to become acid. The same uncertainty obtains as for the pivalate. This compound, an acetate, is likely to bind at the esteratic subsite, and may alkylate a nucleophile in that area. Alternatively, its enzymic hydrolysis may liberate chloromethanol, and then HCl and formaldehyde in the active site, eq. 8, which may inactivate the enzyme.

$$CH_3CO_2CH_2Cl \rightarrow CH_3CO_2H + HOCH_2Cl \rightarrow HCl + CH_2{=}O \qquad (8)$$

Trimethylammoniomethyl acetate, $(CH_3)_3N^+CH_2OCOCH_3$, the lower homologue of AcCh, is a moderately active substrate, with ~1/10 the reactivity of AcCh, and appears to inactivate the enzyme as it hydrolyzes. This also may be a "suicide" inhibitor, due to formation of formaldehyde in the active site:

$$(CH_3)_3N^+CH_2OCOCH_3 \rightarrow CH_3CO_2H + (CH_3)_3N^+CH_2OH \rightarrow$$

$$(CH_3)_3N^+H + CH_2{=}O \qquad (9)$$

tert-Butyl peracetate, $(CH_3)_3COOCOCH_3$, may also act as a "suicide" inhibitor. It has fairly high enzymic reactivity, ~1/10 that of the natural substrate, and leads to partial inactivation as it hydrolyzes, apparently by formation of tert-butyl hydroperoxide, $(CH_3)_3COOH$, in the active site as product of its enzymic hydrolysis. This inactivation is more rapid than that caused by treatment with tert-butyl hydroperoxide. tert-Butyl hypochlorite, $(CH_3)_3COCl$, may merit comparison with the hydroperoxide, but study of esters as potential "suicide" inhibitors would take precedence.

Considering the binding subsite for the β-substituent as trimethyl, rather than anionic, leads to the study of uncharged substrates and inhibitors, which may be tetrasubstituted, but the substituents need not be methyl groups. Thus 2,2,2-trichloroethanol binds similarly to the trimethyl analogues, neopentyl alcohol and tert-butyl methyl ether, and chloral hydrate binds very well, an order of magnitude better than its trimethyl analogue, pivalaldehyde. The binding subsite appears to accommodate the spherical substituents with surface Cl groups as well as those of similar volume with CH_3 surface groups. That the chloral carbonyl favors binding is noteworthy; the reason is not clear and one would hesitate to propose any transition-state analogy. Further, the core atom may be Si instead of C, since the larger $(CH_3)_3Si$ group is accommodated by the active site and leads to reactivity similar to that of the $(CH_3)_3C$ compound.

The methylsulfonyl group, CH_3SO_2, appears to lead to unusual properties and may be of special interest. As a β-substituent in the ester $CH_3SO_2CH_2CH_2OCOCH_3$ (MSAc), it leads to binding similar to that of the analogous CH_3 compound, $(CH_3)_3C$-$CH_2CH_2OCOCH_3$, whether measured as a substrate or as a reversible inhibitor for hydrolysis of AcCh. However, it is substantially less reactive as a substrate. We have attributed this to low effectiveness in removing water from the substrate-enzyme interface in the active site (23). There may, however, be an additional reason. Methanesulfonyl fluoride (MSF), CH_3SO_2F, and related compounds with good leaving groups inactivate AcChE by converting the serine hydroxyl to the methylsulfonyl ester (32), and this action of MSF is accelerated by certain alkyl and aryl cations (33). If the cation accelerates by binding at the trimethyl site, then the CH_3SO_2 is binding at the acetyl subsite, near the serine hydroxyl. Indeed, the $CH_3S(O)$ group in sulfone and sulfoxide is very similar to $CH_3C(O)$. If the cation binds at a peripheral anionic site, this conclusion does not follow, but it remains reasonable. Thus the low reactivity of the sulfonyl substrate MSAc, and of the analogous sulfoxy substrate, Compound IV A (Table II), may arise from non-productive binding, with sulfone or sulfoxy groups interchanged with the ester group in the active site. Similarly, the low reactivity of the amine oxide substrate Compound III (Table II), which has the same binding efficiency as substrate and as reversible inhibitor, may also be due to non-productive binding. Binding of the dimethylamine oxide at the acetyl subsite may resemble that of the dimethylcarbamyl group (34). However, in the three cases, Compounds III, IV and IV A, low reactivity may also be due to inefficient removal of water from the active site.

The sulfonyl group may be of interest as leading to water and lipid permeability and possibly to reversible inhibitors with biological action as moderators of AcChE activity. While methylsulfonyl halides and related compounds are thought to

esterify serine and, thus, not to be useful labeling agents for other parts of the active site, methyl esters of methanesulfonic and benzenesulfonic acids are likely to be methylating agents and thus potential active site-labeling agents. This possibility is being explored, and retardation or acceleration of their action by organic cations will be examined.

Acceleration by organic cations of reactions of AcChE is not restriced to inactivation by methylsulfonyl fluoride. Hydrolysis of small substrates, such as methyl, ethyl and propyl acetates, may also be accelerated in this way (35). When the subsite was considered anionic, this acceleration was attributed to binding of the cations there, thus organizing the active site and rendering it more effective. If the subsite is uncharged, as we propose, uncharged reversible inhibitors of limited size might then also accelerate. In work carried out before the period of this contract, we observed that the simple aromatic compounds nitrobenzene and acetophenone are effective reversible inhibitors, comparable to small organic cations. Our present observation that nitrobenzene and acetophenone accelerate enzymic hydrolysis of ethyl acetate to the same extent as small cations indicates that cationic charge is not necessary, although the electron-attracting character of the substituents may be important for this effect, as it is for the binding. Thus, again, evidence for a specific anionic binding site has not been found. However, it is not clear exactly where these aromatic compounds bind.

With this renewed interest in simple aromatic inhibitors, we have now also observed that styrene epoxide shows irreversible inactivation. This offers an opportunity for developing new reactive agents for labeling the active site.

Table II

Hydrolysis of $X\text{-}CH_2CH_2OCOCH_3$ by Acetylcholinesterase, pH 7.8, 0.18M NaCl, 25°C

Molar Refractivity (MR), Hydrophobicity (π), and Kinetic Constants

Cpd	β-Substituent				$10^9[E]$	$10^{-2}xk_{cat}$	K_m	$10^{-4}x(k_{cat}/K_m)$	$k(HO^-)$	$10^{-4}x(k_{cat}/K_m)_n$
No.	X-	MR cc	π	Conc mM	M	sec^{-1}	mM	$M^{-1}\,sec^{-1}$	$M^{-1}\,sec^{-1}$	$M^{-1}\,sec^{-1}$
I	$(CH_3)_2\overset{+}{S}$-	16.4	-0.50	0.2-1.2	0.6	77	0.33	2300	3.1	2100
I A	$(CH_3)_3\overset{+}{N}$-	17.2	-1.24	0.1-0.6	0.1	160	0.33	4800	2.8	
II	$(CH_3)_3Si$-	25.0	+2.59	0.1-3.0	0.4	57	3.5	160	0.06	7600
II A	$(CH_3)_3C$-	19.6	+1.98	0.7-5.3	0.2	66	5.3	125	0.07	5000
III	$(CH_3)_2\overset{+}{N}(OH)$-	15.0	-3.0	1.5-9.0	0.3	45	18	25	0.82	86
III A	$(CH_3)_2CH$-	15.0	+1.53	1.0-5.0	1.1	34	3.6	93	0.11	2400
IV	$CH_3S(O_2)$-	13.9	-1.9	6-21	0.6	11	6.2	18	1.4	36
IV A	$CH_3S(O)$-	14.1	-1.85	7-22	0.5	38	16	24	0.45	150
IV B	O_2N-	6.7	-0.85	7-22	1.1			(5)		
V	CH_3S-	13.3	+0.45	3-21	0.6	35	15	23	0.28	230
V A	CH_3CH_2-	10.3	+1.02	10-17	0.2	36	13	26	0.11	660
VI	Cl-	5.93	+0.39	4-40	0.2	34	14	25	0.31	220
VI A	Br-	8.80	+0.60	1-12	0.1	45	7.5	59	0.42	400

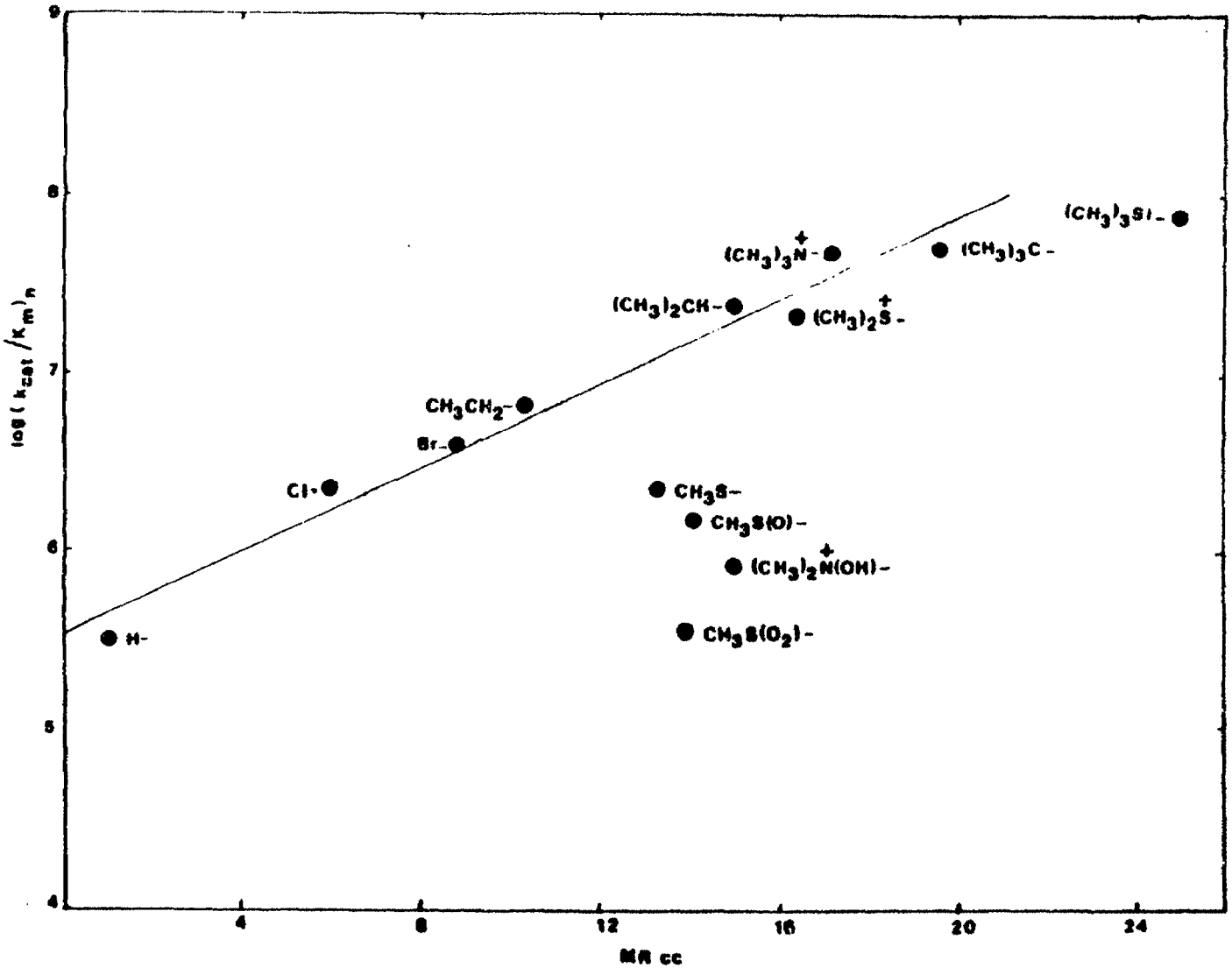


Figure 1. Normalized Reactivity of $X\text{-}CH_2CH_2OCOCH_3$, log $(k_{cat}/k_m)_n$ and Molar Refractivity, $MR_{(X-)}$.

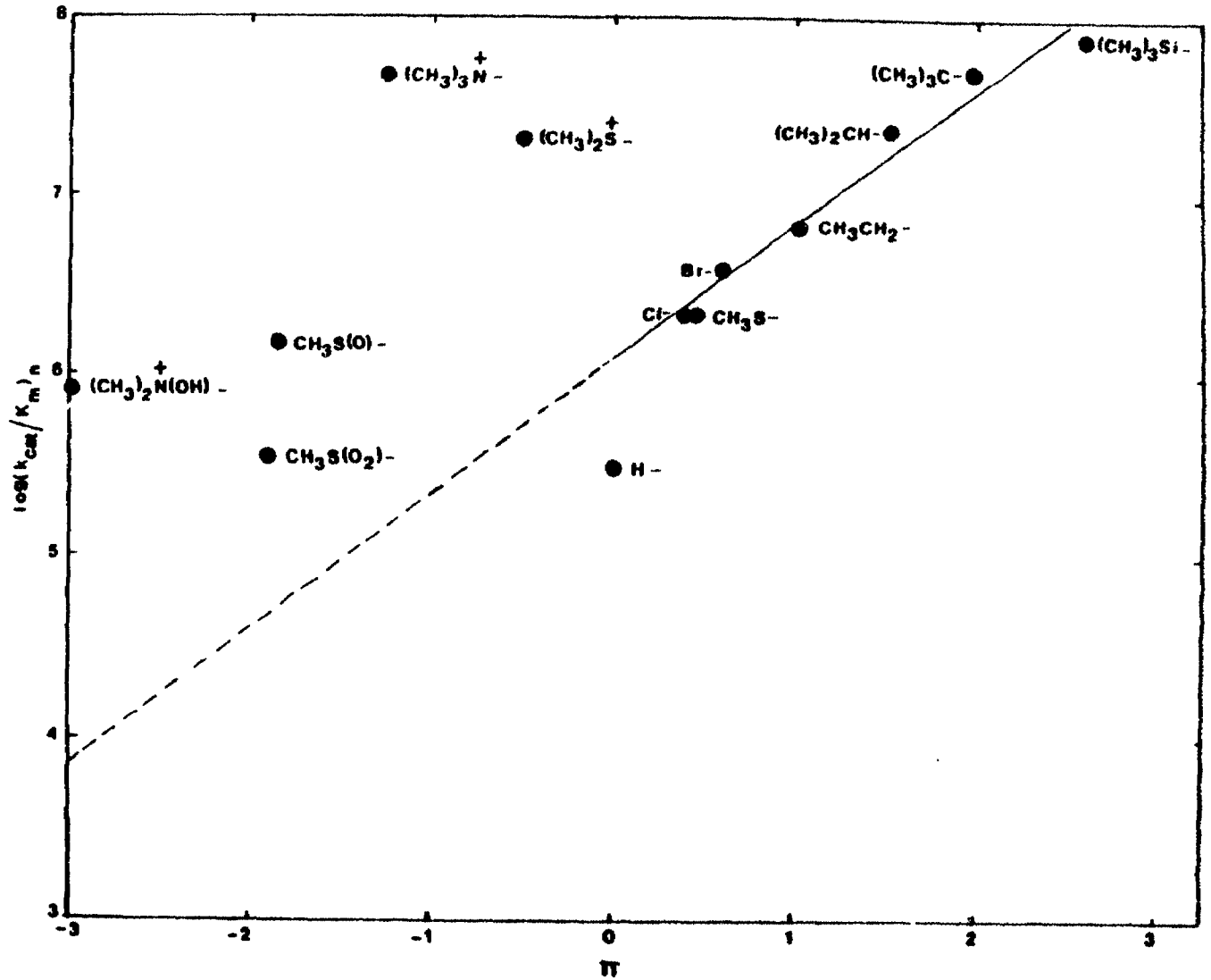


Figure 2. Normalized Reactivity of $X\text{-}CH_2CH_2OCOCH_3$, $\log(k_{cat}/k_m)_n$, and Hydrophobicity $(\pi)_{(X-)}$.

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